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(54) Title: A MIGRATION STIMULATING FACTOR

(57) Abstract

Migration stimulating factor-1 which is a polypeptide capable of stimulating migration of normal adult fibroblasts which do not themselves produce the polypeptide and having an apparent molecular weight of 70kD by polyacrylamide gel electrophoresis, which is cationic at physiological pH, is precipitated from aqueous solution by ammonium sulphate at 10 % saturation or less, which is stable in solution at pH 2 but not at pH 10, is denatured at 56°C and is susceptible to trypsin and alkylation/reduction and which binds heparin. Other migration stimulating factors are similar but anionic. They are produced by foetal or foetal-like fibroblasts from cancer patients (but not normal adult skin fibroblasts) and their production may be used as a diagnostic or prognostic indicator of various cancers.

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A migration stimulating factor.

The present invention relates to novel polypeptides hereinafter referred to as migration stimulating factors (MSF's), to processes for their production and use in diagnosis and therapy.

Cell cultures of various fibroblasts have been investigated and it has been shown that the migratory behaviour of foetal and adult fibroblasts differs and that this difference may be expressed in quantitative terms by the "cell density migration under "(CDM)" (J. Cell. Sci., 73, 221-234 (1985). The inventors have identified a polypeptide which is secreted by foetal fibroblasts and the "foetal-like" fibroblasts of cancer patients and which is involved in mediating the characteristic migratory behaviour of these cells.

This polypeptide has been designated migration stimulating factor-1 or MSF-1. Further investigations have revealed additional polypeptides secreted by some but not all cell lines which secrete MSF-1 and which also have migration stimulating activity. These have been designated as further migration stimulating factors (MSF's). MSF's affect matrix biosynthesis by fibroblasts particularly hyaluronic acid (HA) biosynthesis. This effect on HA biosynthesis is probably responsible for the observed effect of MSF's on fibroblast migration. It is also believed that the MSF's affect protein synthesis by human mammary epithelial cells.

Other polypeptides which promote migratory activity of various cells have been described in the literature. These appear to differ from MSF as explained below: "Scatter Factor" [Stoker, M. and Pennyman, M., J. Cell Sci., 77., 209-224 (1986) and Stoker, M., et al., Nature, 327, 239-242 (1987)] and "autocrine motility factor" (AMF) [Liotta, L. et., Proc. Nat. Acad. Sci., (USA), 83, 3302-3306, (1986)] are distinct from MSF's in that foetal-like breast cancer fibroblasts known to produce MSF-1 do not produce activity in an assay for scatter factor and in that AMF differs from MSF-1 in temperature stability, pH sensitivity, target cell specificity and amino acid composition. Transforming growth factor-β (TGF-β) stimulates HA synthesis in peri-tumour cells but inhibits HA synthesis in corresponding normal cells obtained from reduction mammoplasties whereas MSF-1 stimulates HA synthesis in adult fibroblasts. HA stimulating factor (HASF) is produced by foetal fibroblasts but not by adult fibroblasts but differs from MSF-1 in that MSF-1 is cationic whereas HASF is anionic.

According to the present invention in one aspect there is, therefore, provided a migration stimulating factor.

MSF-1 is a polypeptide having a molecular weight of 70kD by polyacrylamide gel electrophoresis, and the following properties in solution;

TREATMENT	STABLE?
Heat at 56°C	NO
Heat at 100°C	NO
Trypsin	NO
pH 2	YES
pH10	NO
Alkylation/reduction	NO

MSF-1 is precipitated by ammonium sulphate at 10% saturation and binds to a heparin affinity column being eluted at 0.3 and 0.6 M sodium chloride. MSF-1 is believed to have a N-terminal sequence of Ala-Pro-Ile-Pro.

The amino acid composition of MSF-1 from foetal and breast cancer fibroblasts has been analysed as follows:

	RESIDUES PER 100	
amino acid	foetal MSF-1	patient MSF-1
asp+asn	9.0	9.7
flu+gln	12.8	14.1
ser	8.0	8.5
gly	24.4	19.8
his	1.6	2.0
arg	4.1	4.2
thr	4.4	4.7
ala	10.5	10.0
pro	2.2	2.6
tyr	1.4	1.4
val	4.8	5.0
met	-	-
ile	3.1	3.2
leu	5.1	5.2
phe	3.1	2.8
lys	3.9	4.5
cysteic acid	2.2	2.4

MSF-1 is cationic and thus does not bind to anion exchange resins. It binds to cation exchange resins from which it may be eluted using 0.3M sodium chloride solution.

MSF-2 and MSF-3 are anionic, being eluted from anion exchange resins with 0.3 or 3.0M sodium chloride; they do not bind to cationic exchange resins and precipitate from solution with 10 to 20% or 20 to 30% saturated ammonium sulphate.

The present invention therefore further provides a polypeptide capable of stimulating migration of normal adult fibroblasts which do not themselves produce the polypeptide, particularly adult skin fibroblasts, having an apparent molecular weight in the range 50-70 kD by gel filtration and having the solution properties set out above. The polypeptides may be obtained by purification of suitable conditioned media or by synthetic or recombinant DNA techniques. They may be substantially identical to a MSF, a fragment thereof or homologues of a MSF or fragment thereof provided that the migration stimulating activity is retained. Such activity may be assayed readily by techniques described below.

The present invention further provides, in further aspects, (a) a MSF or a polypeptide as hereinbefore defined in solution, preferably at physiological pH and salt concentration, optionally in the presence of buffers, serum, nutrients and/or other components of a cell culture medium and (b) a MSF or a polypeptide as hereinbefore defined in pure, solid form or in solution substantially free of serum, and/or components of cell culture media. In a particularly preferred aspect the invention provides a MSF or a polypeptide as hereinbefore defined in homogeneous form.

The most preferred embodiment of the invention is MSF-1.

The present invention further provides a MSF or a polypeptide as hereinbefore defined for use in a method of treatment of the human or animal body or a method of diagnosis performed on the human or animal body. MSF's and polypeptides as hereinbefore defined have benefits in the diagnosis and treatment of cancer and in wound healing and other disorders involving alterations in fibroblast migration, proliferation, biosynthetic activity and ability to interact with other cell types, in particular epithelial cells.

The invention further provides a pharmaceutical composition comprising a MSF or a polypeptide as hereinbefore defined together with a diluent or carrier therefor and optionally further comprising stabilisers, anti-oxidants, anti-biotics such as anti-bacterials and/or anti-fungals, buffers, salts and the like.

The invention further provides a method for the treatment of the human or animal body comprising administering an effective non-toxic amount of a MSF or a polypeptide as hereinbefore to a patient in need thereof.

Administration may be by any suitable route and in any pharmacologically acceptable form adapted to administration by that route and in sufficient amount to achieve a desired effect, for instance on migration of fibroblasts or alteration in fibroblast biosynthetic activity.

The invention further provides the use of a MSF or a polypeptide as hereinbefore defined in the preparation of a medicament for use in a method of treatment of the human or animal body by therapy or in a method of diagnosis practiced on the human or animal body.

The present invention further comprises a test method or assay which comprises contacting MSF-sensitive cells, which do not secrete MSF's, preferably adult fibroblasts with a sample suspected to contain a MSF or a polypeptide as hereinbefore defined. The presence and/or concentration of a MSF or a polypeptide as hereinbefore defined in for instance, a culture medium or sample of a body fluid such as whole blood or serum, is detected and/or determined for instance by observing the migration behaviour of the cells (preferably by measurement of their CDMI) and optionally comparing this behaviour with standards. The MSF-sensitive cells are preferably normal adult skin fibroblasts and are preferably in the form of a confluent culture on a 3-dimensional collagen matrix. The samples may be fractionated, diluted or concentrated in order to adjust the concentration of MSF or polypeptide therein to optimise the response of the cells. Preferably the test or assay is conducted in solution for instance in a buffer and most preferably the solution has a concentration of MSF or polypeptide of about 10ng/ml.

In one aspect of this test method or assay the sample is a cell culture medium or a fraction thereof such as a solution of a partially purified MSF or a polypeptide as hereinbefore

defined and the test or assay is conducted to determine whether cells are able to produce a MSF or a polypeptide as thereinbefore defined (and therefore whether they are "foetal-like" with respect to this particular characteristic; such aberrant fibroblasts in a tissue sample would suggest increased susceptibility to developing cancer). This method may also be used as a part of a purification process for obtaining a MSF or a polypeptide as hereinbefore defined from cell culture or in order to identify fractions containing MSF or a polypeptide.

In a second aspect of this test method or assay the sample is a body fluid such as serum and the assay is conducted as an aid to diagnosis or prognosis of cancer; for instance elevated circulating levels of a MSF or a polypeptide as hereinbefore defined may be associated with increased risk of developing cancer or disease prognosis in individuals already having cancer.

In a third aspect of the test method or assay the sample may be conditioned medium prepared by culturing fibroblasts from a cancer patient or from an individual suspected to be at risk from cancer such as breast cancer and the test or assay may be conducted as an aid to diagnosis or prognosis thereof.

MSF's and polypeptides as hereinbefore defined may be used to raise antibodies by conventional immunisation techniques. The antibodies (polyclonal or monoclonal) are a further aspect of the invention. They may also be used in test or assay techniques, such as radio-immunoassay (RIA) or enzyme linked immunosorbent assay (ELISA) techniques for detecting a MSF or a

polypeptide in a sample such as a culture medium or body fluid. Labelled antibodies and test or assay methods using antibodies against a MSF or a polypeptide are a further aspect of the invention.

The invention further provides a process for producing a MSF or polypeptide as hereinbefore defined, which process comprises culturing fibroblasts capable of secreting MSF, such as foetal fibroblasts, foetal-like fibroblasts of cancer patients or other MSF-secreting fibroblasts found in the normal adult such as oral mucosal fibroblasts capable of expressing MSF in a suitable culture medium and recovering the conditioned medium so produced. Adult skin fibroblasts do not secrete MSF's whereas oral mucosal fibroblasts appear to do so, consistent with the foetal-like mode of wound repair in the oral mucosa. Preferably the process further comprises purification steps such as gel filtration and/or reverse phase chromatography and/or additional conventional purification steps.

In order to purify MSF or a polypeptide as hereinbefore defined from conditioned media it is necessary to establish a bioassay or other assay technique for identifying fractions containing the MSF or a polypeptide as hereinbefore defined. A suitable bioassay technique is that described in Example 1 below using FSF 37 cells or any other fibroblasts highly sensitive to the presence of MSF or polypeptide as hereinbefore defined. Highly sensitive cells can be identified by screening fibroblasts and fibroblast cell lines using a foetal or transformed fibroblast conditioned medium containing a MSF or a

polypeptide as hereinbefore defined and selecting those cells or that cell line which demonstrates a sufficiently high or the highest response in terms of increased migratory behaviour.

The present invention further provides a process for increasing the migration of fibroblasts comprising contacting fibroblasts with a MSF or a polypeptide as hereinbefore defined.

In further aspects of the invention there are provided: antibodies against a MSF or a polypeptide as hereinbefore defined; RNA and DNA fragments encoding a MSF or a polypeptide as hereinbefore defined; genes comprising regulatory DNA sequences and DNA sequences encoding MSF or a polypeptide as hereinbefore defined; cloning and/or expression vectors comprising DNA or genes as hereinbefore defined; cells transformed with such cloning or expression vectors and nucleic acid probes for detecting RNA or DNA fragments or genes as hereinbefore defined.

The invention will now be illustrated by the following non-limiting Examples and with reference to the Figures of the accompanying drawings in which:

Figure 1: Scatter diagram of migration values of normal adult, fetal and breast cancer patient (BSF) skin fibroblasts plated at high and low cell densities.

Figure 2: Dose-response data concerning the effects of fetal (0-0) and BSF (●-●) conditioned medium on the migration of a foreskin fibroblast target line (FSF37) plated at high density.

Figure 3: Fetal fibroblast (FS6) CM was fractionated by gel filtration chromatography and the collected samples then tested for their ability to stimulate the migration of FSF37 target cells. As in the standard migration assay, the FS6 CM fractions were tested at a final concentration of 25% in medium containing 5% serum. The first bar (labelled "C") is the level of FSF37 migration achieved in control cultures in which SF-MEM was used instead of the FS6 CM fractions.

Figure 4: The effects of fetal and BSF CM on the proliferation of fetal (FS6) and foreskin (FSF37) fibroblasts. Cells were plated in 35 mm plastic tissue culture dishes at 2×10^4 cells per dish in medium containing 5% serum (X-X), or this medium supplemented with 25% fetal (0-0) or BSF (o-o) CM. The fetal fibroblasts achieved a higher plateau cell density (4.9×10^5) than the adult cells (1.4×10^5). Neither fetal nor BSF CM affected cell proliferation.

EXAMPLE 1

We have previously reported that (a) the migration of fetal and adult fibroblasts into three-dimensional collagen matrices is differentially affected by cell density, and (b) skin fibroblasts from cancer patients commonly display a fetal-like mode of migratory behaviour. Data presented here indicate that differences in the migration of these cell types are particularly apparent in cultures plated at high density (ie. at cell confluence): under these conditions, fetal fibroblasts and the fetal-like fibroblasts or cancer patients migrate into the three-dimensional collagen matrix to a significantly greater extent than do normal adult cells. In this initial study concerned with the biochemical basis of these observations, we report that medium conditioned by either fetal or cancer patient fibroblasts stimulates the migration of confluent adult cells. This stimulation of migration is specific to confluent cells, as the migration of subconfluent adult fibroblasts is unaffected by these conditioned media. Gel filtration chromatography of fetal fibroblast conditioned medium indicates that migration stimulating activity is recovered in a single peak with an apparent molecular mass in the range of 50-60 kD. The active migration stimulating factor (MSF) in both fetal and cancer patient fibroblast conditioned media appears to be a protein stable at acid pH, but inactivated by heat, alkaline pH and reductive alkylation. MSF produced by fetal and cancer patient fibroblasts is presumably responsible for the characteristically elevated levels of migration displayed by these cells in

confluent culture, thereby suggesting an autocrine mode of action for this factor. Stimulation of adult cell migration by MSF requires the presence of either serum or platelet-poor plasma and is not observed in serum-free medium. MSF does not appear to affect either the proliferation of morphology of normal adult cells under any of the culture conditions examined.

In spite of their wide-spread tissue distribution and relative ease with which they are cultured, fibroblasts remain a rather poorly defined group of cells commonly identified in vitro on the basis of such non-specific attributes as spindle-shaped morphology. Recent evidence has served to underscore the fact that fibroblasts are in fact a highly heterogeneous family of cells displaying both developmental and site-specific differences in such fundamental aspects of cell behaviour as proliferation, response to growth factors and matrix biosynthesis (Schor and Schor, 1987a). This heterogeneity in fibroblast phenotype is presumably of fundamental importance to the normal functioning of connective tissues, including their inductive interactions with neighbouring epithelia.

When dermal fibroblasts are plated on the surface of three-dimensional gels of type I collagen fibres, they begin to migrate down into the underlying gel matrix within 24 hr. This aspect of fibroblast migratory behaviour (ie. from the gel surface to interior) is influenced by a number of experimental parameters, including cell density on the gel surface (Schor et al, 1982). The effect of cell density on fibroblast migration may be expressed in quantitative terms by a function we have defined as the "cell density migration index" or "CDMI" (Schor et al, 1985a). Using this experimental approach, we have previously reported that

(a) fetal and normal adult skin fibroblasts may be distinguished on the basis of the distinctive CDMI values they express (Schor et al, 1985a) and (b) ostensibly normal skin fibroblasts from

patients with cancer commonly express CDMI values falling within the fetal range (Durning et al, 1984: Schor et al, 1985b: Schor et al, 1986; Haggie et al, 1987). On the basis of these findings, we put forward an hypothesis suggesting that the dysfunction in normal epithelial-mesenchymal interactions caused by the persistence of fetal-like fibroblasts in the adult leads to an increased susceptibility to the development of cancer (Schor et al, 1987).

The CDMI is specifically a measure of the effects of cell density on migration. This differential effect of cell density may be mediated by a number of mechanisms, including (a) cell-induced alterations in the orientation and/or packing density of collagen fibres in the gel (Grinnell and Lamke, 1984), (b) density-dependent changes in the deposition of specific matrix macromolecules (eg. fibronectin) known to affect cell migration (Mautner and Hynes, 1977; Schor et al, 1981), (c) social interactions between cells (Abercrombie, 1970), and (d) the secretion of a migration stimulating or inhibiting soluble factor. These different possibilities have been discussed in a recent review (Schor and Schor, 1987b).

The objective of the present study is to learn more about the biochemical basis of the different CDMI values displayed by fetal, normal adult and cancer patient skin fibroblasts. Our data suggest that fetal fibroblasts and the fetal-like fibroblasts of cancer patients secrete an autocrine migration stimulating factor not produced by normal adult cells.

MATERIALS AND METHODS

cells and culture conditions

Fibroblast lines were established from explant cultures, as described by Ham (1980). FSF37 cells were derived from a foreskin biopsy of a 6 year old child. Further details regarding the normal adult and fetal skin fibroblasts used in this study may be found in Schor et al (1985a); similar data regarding the breast cancer patient skin fibroblasts (BSF) are presented in Haggie et al (1987).

Stock cultures of skin fibroblasts were grown in 90mm plastic tissue dishes in MEM, growth medium (Gibo-BioCult, Scotland) supplemented with 15% aseptic newborn calf serum, glutamine, non-essential amino acids, sodium pyruvate, penicillin and streptomycin (Schor and Court, 1979). Cultures were passaged at a split ratio of 1:5 approximately once a week when confluence was achieved.

Where indicated, bovine plasma and serum were prepared as described by Vogel et al (1978).

collection and treatment of conditioned media

Confluent stock cultures were washed 5 times with serum free growth medium (SF-MEM) and then incubated with 5ml SF-MEM for 72 hr at 37°C in a humidified gassed incubator. The resultant conditioned medium (CM) was then collected, passed through a 0.22u Millipore filter to remove cellular debris and stored at -70°C until required.

Where indicated, samples of CM were treated as follows:

(a) dialysis: against 100x volume of SF-MEM for 24 hr at 4°C;

(b) trypsinization: 2.5% solution of crystalline trypsin (Sigma Chemical Co., T-0134) in calcium and magnesium free Dulbecco's phosphate buffered saline (PBS) added to give a final concentration of 0.25% trypsin; after incubation for 10 min at 37°C, the trypsin was inactivated by addition of soybean trypsin inhibitor (Sigma Chemical Co., T-9003); (c) heated: to either 60° or 100°C for 10 min; (d) pH stability: CM adjusted to either pH 2 with 1N HCl or pH 10 with 1N NaOH, incubated for 60 min at 4°C and then readjusted to pH 7.2: (e) reductive alkylation: CM treated with 20mM mercaptoethanol for 24 hr at room temperature, followed by addition of iodoacetic acid (18 ug/ml) and incubation at 40°C for 1 hr; the pH was then adjusted to 7.5 with solid Tris-base and the treated sample dialysed against SF-MEM for 24 hr.

Samples of fetal fibroblast (FS6) CM were concentrated tenfold using an Amicon filtration cell fitted with a YM5 membrane (molecular weight cut off 5000 kD). Neat or concentrated CM was fractionated by gel filtration chromatography using a Pharmacia fast protein liquid chromatography system (FPLC^{tm}). 200 ul of CM was applied to a Superose 12 column and fractionated at a flow rate of 0.3 ml/min in 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl. 1 ml fractions were collected and then dialysed against SF-MEM for 48 hr at 4°C. The column was calibrated by running a mixture of molecular weight standards (B-amylase, 200 kD; alcohol dehydrogenase, 150 kD; bovine serum albumin, 66 kD; carbonic anhydrase, 29 kD; cytochrome C, 12.4 kD).

cell growth and migration assay

Cell proliferation was measured as previously described (Schor, 1980). Cells growing on 35mm plastic tissue culture dishes were brought into a single cell suspension by treatment with 0.05% trypsin in PBS containing 2 mM ethyleneglycol-bis (b-aminoethylether)-N,N' -tetraacetic acid (EGTA). Cell number was measured with a Coulter electronic particle counter.

Type I collagen was extracted from rat tail tendons in 3% acetic acid, dialysed for two days against distilled water and used to make 2 ml collagen gels in 35 mm plastic tissue culture dishes as described previously (Schor and Court, 1979). For the migration assays, collagen gels were overlaid with 1 ml of either SF-MEM or CM. Fibroblasts growing in stock culture were trypsinized and resuspended in growth medium containing 20% serum. This cell suspension was then used to prepare two plating inocula (high and low density, respectively). The cell count in the high density inoculum was adjusted so that plating 1 ml gave 2.5×10^4 cells cm^{-2} on the gel surface; the count in the low density inoculum was similarly adjusted to give 10^3 cells cm^{-2}. Fibroblasts attached and were fully spread on the gel surface within 2 hours after plating; cells in the low density cultures were sparsely distributed on the gel, whilst cells in the high density cultures formed a confluent layer immediately upon spreading. Considering the 2 ml volume of the hydrated collagen gel, this procedure gives final concentrations of 5% serum and 25% CM in the standard migration assay. Replicate high and low density cultures were incubated for 4 days and the number of cells on the gel surface and within the

three-dimensional collagen matrix then determined by microscopic observation of living cultures using phase optics (Schor. 1980). Data are expressed as the percentage of cells found within the gel matrix. A minimum of 1000 and 1500 cells were counted to determine each percentage point for cells plated at low density and high density respectively.

CDMI determinations

The "cell density migration index" or "CDMI" may be used to express the effects of cell density on fibroblast migration in quantitative terms (Schor et al, 1985a). The CDMI is defined as follows:

CDMI = log [migration low density/migration high density]

where "migration low density" is the percentage of cells within the gel matrix in low density cultures and "migration high density" is the corresponding value for high density cultures. On the basis of results obtained with a large number of well characterized normal adult, foreskin, fetal and overtly transformed cell lines, we have previously defined four ranges of CDMI values (Schor et al, 1985a); ie. the transformed range (T) with CDMI values less than -0.4, the transformed/fetal range (T/F) with CDMI values between -0.4 and 0, the fetal/normal range (F/N) with CDMI values between 0 and +0.4 and the normal range (N) with CDMI values greater than +0.4. These four ranges were empirically derived so that greater than 90% of normal adult and foreskin fibroblasts had CDMI values falling in the N range, whilst greater than 90% of fetal cells had CDMI in the F/N and T/F ranges. For clarity in this communication, the N

range is renamed A (for normal adult), whilst the the T/F and F/N ranges are collectively referred to as the F range (for fetal).

RESULTS

the differential effect of cell density on the migration of adult, fetal and breast cancer patient skin fibroblasts

Fibroblasts were plated on the surface of collagen gel substrata at 10^3 cells cm^{-2} (low density) and 2.5×10^4 cells cm^{-2} (high density) and cell migration into the gel then measured as described in Materials and Methods. Data obtained with 40 lines of adult skin fibroblasts, 26 lines of fetal skin fibroblasts and 15 lines of breast cancer patient skin fibroblasts (BSF) are presented in Figure 1. The migration of adult skin fibroblasts was inversely proportional to cell density, with median values of 24.6% cells within the gel in low density cultures and 5.9% in high density cultures. In contrast, the migration of the fetal cells was not significantly influenced by cell density, with median values of 16.5% and 15.8% in the low and high density cultures, respectively. Corresponding data for the BSF cells gave median values of 25.3% (low density cultures) and 13.2% (high density cultures). These results indicate that fetal and BSF fibroblasts in high density culture migrate to a significantly greater extent than normal adult cells ($p < 0.001$ in both cases, Student's t test).

CDMI values for the individual cell lines were calculated from these results. 36/40 (90%) of the adult fibroblast lines gave CDMI values falling in the A range, 24/26 (92%) of the fetal fibroblasts were in the F range and 13/15 (87%) of the BSF

cells were in the F range.

effects of conditioned media on cell migration

The possible involvement of a cell produced soluble factor in determining the characteristic migratory behaviour of the adult, fetal and BSF fibroblasts was investigated in the following fashion. Foreskin (FSF37) and fetal (FS6) fibroblasts were plated onto replicate collagen gels at both high and low densities in growth medium containing a final concentration of 5% serum, with or without 25% conditioned medium (CM) from foreskin, fetal or BSF skin fibroblasts. The percentage of cells present within the collagen matrix was measured after 4 days incubation. Data presented in a previous study (Schor et al, 1985a) indicated that foreskin fibroblasts (obtained from donors with ages ranging between 2-8 years) were indistinguishable from normal adult cells (both male and female, obtained from a variety of anatomical sites) in terms of their migratory behaviour on collagen gels. As can be seen in Table 1, the FSF37 foreskin fibroblasts displayed a characteristically adult-like migratory response to cell density in control cultures incubated in the absence of CM; under these conditions, 20.8% of the cells were within the gel matrix in low density cultures compared to only 2.0% in high density cultures, with a resultant CDMI of +1.02 (A range). The presence of fetal CM had no significant effect on FSF37 cell migration in low density cultures (18.6%), but resulted in an approximate 6-fold stimulation of migration in high density cultures (12.7%); these cells now displayed a chacteristically fetal-like pattern of migration, with a calculated CDMI of +0.16 (F range).

matrix / target cell	CM	% of cells within gel: low density	high density	CDMI
adult (FSF37)	none	20.8	2.0	+1.02 (A)
	FS6	18.6	12.7	+0.16 (F)
(A)	FSF37	27.8	3.9	+1.14
(F)	BSF11	19.7	13.9	+0.15
fetal (FS6)	none	15.3	14.8	+0.01 (F)
	FS6	11.1	10.5	+0.02 (F)
	FSF37	15.9	12.6	+0.10 (F)
	BSF11	14.8	15.0	-0.01 (F)

TABLE 1: THE EFFECTS OF CONDITIONED MEDIUM ON FIBROBLAST MIGRATION

Conditioned media (CM) were prepared by incubating confluent cultures of the different fibroblast lines in serum-free growth medium for three days. The migration assays were carried out in the presence of 5% serum and 25% CM. The migration data obtained in high and low density cultures were used to calculate the respective CDMI values; A = normal adult range and F = fetal range.

BSF CM had a similar effect to that of fetal CM on the migration of FSF37 cells, giving values of 19.7% and 13.9% cells in the gel matrix in low and high density cultures, respectively (calculated CDMI of +0.15 in F range). The presence of FSF37 CM had no effect on the migration of FSF37 cells at either high or low cell densities.

In the reciprocal experiment, FS6 fetal fibroblasts displayed characteristically fetal-like migratory behaviour under all culture conditions. None of the CMs examined had a significant effect on cell migration at either high or low cell density. It should be noted that FSF37 fibroblast CM did not inhibit the migration of fetal fibroblasts in high density culture. The migration of BSF cells at both high and low cell densities was similarly unaffected by all of the CMs examined.

The effect of CM produced by a number of other normal adult, fetal and BSF skin fibroblast lines on the migration of FSF37 cells in high density culture are presented in Table 2. None of the adult CMs had any demonstrable effect on cell migration. In contrast, CMs produced by all of the fetal and BSF lines induced a significant stimulation of migration. None of these CMs affected the already high levels of migration of FSF37 cells in low density cultures (data not presented)

initial characterization of the fetal and BSF migration stimulating factor

The above results suggest that fetal and BSF skin fibroblasts produce a soluble factor that stimulates the

source of CM	% of FSF37 cells in gel	relative stimulation
(a) control no CM	2.2	
(b) fetal		
FS6	13.1	5.9
FS2	12.9	5.8
FS8	15.9	7.2
FS13	13.4	6.0
FS10	17.9	8.1
FS12	9.2	4.2
		mean = 6.2 +/- 1.3
(c) adult		
FSF37	1.2	0.6
FSF36	2.1	0.9
NSF28	2.9	1.3
NSF30	2.4	1.1
NSF111	2.2	1.0
NSF130	2.3	1.1
		mean = 1.0 +/- 0.2
(d) breast cancer patient		
BSF7	8.7	3.9
BSF11 PS	12.6	5.7
BSF30	10.1	4.6
BSF31 SE	10.7	4.8
BSF37 EWT	6.7	3.1
		mena = 4.4 +/- 1.0

TABLE 2: THE EFFECTS OF DIFFERENT FIBROBLAST CONDITIONED MEDIA ON THE MIGRATION OF FSF37 CELLS

Data are presented concerning the effects of conditioned media produced by different fetal, normal adult and breast cancer patient skin fibroblasts on the migration of a target foreskin fibroblast line (FSF37) in high density culture. The mean +/- SD for each class of fibroblast (fetal, adult and cancer patient) are also given.

migration of adult fibroblasts in high density culture, but has no effect on cell migration in low density culture Data concerned with the initial characterization of the active migration stimulating factor (MSF) in fetal and BSF CM are presented in Table 3. Fetal and BSF CMs were exposed to a variety of treatments and their ability to stimulate the migration of FSF37 fibroblasts in high density culture were then examined. The results indicate that the migration stimulating activity in both fetal and BSF CM is non-dialysable, acid-stable and inactivated by heat, trypsin, exposure to pH10 and alkylation reduction. These data are consistent with MSF being a protein containing disulphide bonds required for its activity. The migration of FSF37 cells in low density culture was not affected by CMs treated by any of the above procedures.

Dose-response data for the stimulation of FSF37 fibroblast migration by fetal and BSF CMs are presented in Figure 2. There was an approximately linear response to CM concentrations between 1-25%, after which point a plateau level of stimulation was achieved. Both fetal and BSF CMs gave identical results. The maximal stimulation of FSF37 migration (13.3%) is within the range of that displayed by the fetal and BSF cells themselves in high density culture (see Figure 1).

gel filtration chromatography of fetal fibroblast CM

Neat CM produced by FS6 fetal fibroblasts was fractionated by gel filtration chromatography as described in Materials and Methods. Individual fractions w re then tested for their ability to stimulate the migration of FSF37 fibroblasts plated at high

	% of FSF37 fibroblasts in gel matrix		
	fetal CM		BSF CM
	low density	high density	high density
(a) control (ie. no CM)	23.7	1.7	1.7
(b) treatments			
none	22.7	13.3	12.6
56°C	21.8	4.8	3.3
100°C	22.8	1.7	1.5
trypsin	20.6	0.5	3.0
dialysis	25.1	12.4	10.2
pH 2	19.7	8.6	16.6
pH 10	22.2	1.9	5.5
alkylation/ reduction	24.8	2.9	3.2

TABLE 3: SENSITIVITY OF FETAL BSF CM TO DIFFERENT TREATMENTS

CM obtained from fetal (FS6) and breast cancer patient (BSF11) fibroblasts were exposed to the various treatments indicated in the Table and then used in a standard migration assay using FSF37 fibroblasts as the target cell line. The effects of fetal CM were ascertained at both high and low plating densities, while BSF CM was examined only in high density culture.

cell density in our standard assay system. As can be seen in Figure 3, a single peak of migration stimulating activity was recovered in fractions 11 and 12, corresponding to an apparent molecular weight in the range of 50-60 kD. Identical results were obtained following the fractionation of 10-fold concentrated FS6 CM (data not shown).

the effects of fetal and BSF CM on fibroblast proliferation

The effects of fetal and BSF CM on the proliferation of fetal (FS6) and adult (FSF37) fibroblasts are shown in Figure 4. Cells were plated in 35mm plastic tissue culture dishes at 2 x 10^4 cells per dish in medium containing 5% serum and 25% of the indicated CM. The fetal fibroblasts achieved a higher saturation density (4.9 x 10^5 cells/dish) than the adult (1.4 x 10^5 cells/dish). Neither fetal nor BSF CM had any effect on the proliferation of these cells. The growth of FSF 37 cells on or within three-dimensional collagen gel substrata was similarly unaffected by both fetal and BSF CM (data not shown).

the stimulation of adult fibroblast migration by MSF is dependent upon serum or plasma

Cell migration is known to be affected by a number of well-characterised soluble growth factors; eg. platelet derived growth factor, PDGF (Bernstein et al, 1982) and epidermal growth factor, EGF (Blay and Brown, 1985). These factors are present in different quantities in serum and plasma. With this rationale in mind, we examined the effects of serum and plasma on the ability of fetal and breast cancer patient fibroblast

conditioned medium to stimulate the migration of FSF37 target foreskin cells.

In the experiment presented in Table 4, cells were plated at high density in the presence or absence of the different conditioned media in (a) serum-free medium, (b) medium supplemented with 5% bovine plasma and (c) medium supplemented with 5% bovine serum. Our results indicate that cell migration in the absence of conditioned medium occurs to the same extent in serum-free medium and medium supplemented with either serum or plasma. These results are consistent with previous reports indicating that the migration of several cell types, including human skin fibroblasts and melanoma cells, into collagen gels is not dependent upon the presence of serum (Schor et al, 1981). In contrast, stimulation of FSF37 migration in high density cultures by either fetal or breast cancer patient conditioned medium] was only observed in the presence of serum of plasma; cells cultured in serum-free medium were completely unresponsive to these conditioned media.

DISCUSSION

The data presented here indicate that (a) fetal and BSF fibroblasts migrate to a significantly greater extent in high density culture than do normal adult cells, (b) fetal and BSF fibroblasts produce a soluble factor (MSF) which stimulates the migration of normal adult cells in high density culture (with a consequent expression of CDMI values falling in the f tal range), (c) fetal and BSF CM do not affect the migration of adult cells in low density culture, and (d) adult fibroblast CM

	% of cells in gel matrix		
conditioned medium	serum-free	plasma	serum
none	4.6	2.3	4.9
fetal	3.9	8.4	10.8
breast cancer	4.6	9.4	11.1

TABLE 4: THE EFFECTS OF SERUM AND PLASMA ON THE STIMULATION OF FIBROBLAST MIGRATION BY FETAL AND BREAST CANCER PATIENT FIBROBLAST CONDITIONED MEDIA

FSF37 foreskin fibroblasts were plated onto collagen gels at high density in the presence and absence of fetal (FS6) and breast cancer patient (BSF11) fibroblast conditioned media in (a) serum-free medium, (b) medium containing 5% bovine plasma and (c) medium containing 5% bovine serum. The percentage of cells within the gel matrix was measured after four days of incubation.

does not inhibit cell migration. The differential effect of the factor on the migration of FSF37 cells at high and low densities is consistent with the potential role of this factor in determining the distinctive CDMI values displayed by the different classes of fibroblasts.

The migration stimulating activity in fetal and BSF CM displayed the same sensitivity to various treatments (trypsinization, dialysis, heat, pH change, reductive alkylation). The similarity of fetal and BSF migration stimulating activity is again indicated by the identical dose-response curves of the respective CMs. Further characterization and purification of the respective factors will reveal whether fetal and breast cancer patient fibroblast MSF are in fact identical. Gel filtration chromatography of fetal fibroblast CM indicates that MSF has an apparent molecular mass in the range of 50-60 kD. Identical results have been obtained for MSF produced by other fetal and breast cancer patient fibroblasts; these data will be presented in a subsequent communication (Grey et al, in preparation), in which further details are given regarding the purification and biochemical characterization of MSF.

For various purposes (including the purification of MSF), it will be convenient to express migration stimulating activity in terms of units per ml CM or units per ug protein. We have therefore defined a unit of activity as that required to produce a 3-fold stimulation in the migration of a target adult fibroblast in high density culture. Examination of the dose-response data presented in Figure 2 indicates that a migration

level corresponding to one unit of migration stimulating activity is achieved at a concentration of 10% CM. Since both fetal and BSF CM contain approximately 50ug protein per ml, we calculate that these CMs contain in the region of 10 units per ml of 0.2 units per ug protein.

Neither fetal nor BSF CMs stimulated adult cell proliferation: these results indicate that the ability of MSF to stimulate fibroblast migration is not dependent upon the induction of cell proliferation. In this regard, recent data suggest that the stimulation of adult fibroblast migration by MSF is a secondary consequence of its primary effect upon the deposition of matrix macromolecules, in particular hyaluronic acid (Schor and Schor, 1987b). This finding is in keeping with the growing body of evidence indicating that many soluble "growth" factors, eg. transforming growth factor-beta, exert a primary effect upon matrix synthesis (Ignotz and Massague, 1986).

The results presented in Table 4 suggest that the mechanism by which the MSF stimulates cell migration is dependent upon the concerted action of other factors present in either serum or plasma. These results would appear to exclude a dependence upon PDGF and other platelet derived factors since these are not present in platelet-poor plasma. It should also be noted that in contrast to this absolute dependence upon serum or plasma for MSF action, it is nonetheless produced by both fetal and breast cancer patient fibroblasts under serum-free conditions; ie. our standard method for obtaining conditioned medium.

Migration stimulating factors produced by a variety of cell types and displaying a diversity of target cell specificities have recently been described. It is of interest to note that many of these factors have apparent molecular masses in the range of 50-60 kD (Stoker and Pennyman, 1986; Liotta et al, 1986; Stoke et al, 1987; Atnip et al, 1987). A number of lines of evidence, however, suggest that they are not identical with fibroblast MSF. For example, fetal fibroblasts produce a scatter factor which induces the dispersion of normal epithelial cells from their characteristically tightly packed colonies (Stoker and Pennyman, 1986; Stoker et al, 1987). Using this assay system, it was found that FS6 fetal fibroblast CM contained measurable scatter factor activity, whilst BSF11 breast cancer patient fibroblast conditioned medium was negative (Stoker et al, 1987; and personal communication). These results suggest that the epithelial scatter factor described by Stoker (present only in fetal fibroblast CM) and the fibroblast migration stimulating factor discussed here (present in both fetal and BSF CM) may not be the same biochemical entity. In addition, scatter factor acts in a paracrine fashion, since it is produced by fetal fibroblasts, but exerts its biological activity on normal epithelial cells; in contrast, the migration stimulating factor described in this communication is assayed by virtue of its effect upon target adult fibroblasts. The elevated levels of migration displayed by fetal and BSF fibroblasts in high density culture most probably result from the constitutive production of MSF by these cells, thereby suggesting an autocrine mode of action for this factor.

Migration stimulating factors have also been reported to be produced by metastic tumour cell lines. Autocrine motility factor (AMF) described by Liotta et al (1986) is perhaps the best characterized. Our preliminary data suggest that AMF is not the same molecule as fibroblast MSF; significant differences between the two factors include (a) heat stability (MSF inactivated by heating to 60°C, whilst AMF is not), (b) pH sensitivity (MSF stable at acid pH and labile at alkaline pH, whilst AMF labile at acid pH and stable at alkaline pH), (c) dependence upon serum or plasma (MSF requires the presence of either serum or plasma for activity, whilst AMF is assayed in serum-free medium), and (d) target cell specificity (MSF stimulates the migration of non-producing normal adult target cells, whilst AMF stimulates the migration of producing metastatic tumour cell lines, but not normal or non-metastatic cell lines).

It should also be emphasized that the activities of these various migration stimulating factors are assayed under quite different conditions; MSF is identified by its stimulation of fibroblast migration into three-dimensional collagen gels during an extended incubation period of 4 days, scatter factor by its dispersion of epithelial cells growing on plastic dishes and AMF by its effect upon cell migration into a Nucleopore filter during an incubation period of only 4 hours. Such fundamental differences in the assay procedure must be born in mind when comparing different factors which aff ct such a complex behavioural attribute as cell migration. In spite of these important caveats, the apparent similarity in molecular weight

displayed by these various factors (50-60 kD) raises the intriguing possibility that they may nonetheless be members of a related family of molecules.

We have previously discussed the possibility that the presence of fetal-like fibroblasts in the adult increases susceptibility to the development of cancer by virtue of the resultant dysfunction in normal epithelial-mesenchymal interactions (Schor et al, 1987). In the present study we have demonstrated that the fetal-like fibroblasts of cancer patients secrete a migration stimulating factor with similar biochemical properties to that produced by bone fide fetal cells. A more detailed consideration of the fetal-like fibroblasts of cancer patients and their potential involvement in disease pathogenesis is presented in the accompanying communication.

EXAMPLE 2

MATERIALS AND METHODS

cells and culture conditions

Fibroblast lines were established from explant cultures, as described by Ham (1980). The cell lines used in this study were: FSF37 foreskin fibroblasts obtained from a 6 year old male donor; FS6 fetal limb dermal fibroblasts obtained from a 12 week female fetus; BSF11 forearm dermal fibroblasts obtained from a 50 year old female patient with familial breast cancer. The FSF37 foreskin fibroblasts display a characteristically adult pattern of migratory behavior and do not produce MSF; these cells will therefore be referred to as adult fibroblasts in this communication. Stock cultures were grown in 90 mm plastic tissue culture dishes in MEM growth medium supplemented with 15% aseptic newborn calf serum, glutamine, non-essential amino acids, sodium pyruvate, penicillin and streptomycin (Schor and Court, 1979). Cultures were passaged at a split ratio of 1:3 approximately once a week when confluence was achieved. Fibroblasts between passages 5-10 were used in this study. Cells used for experiments were free of mycoplasmal contamination, as assessed by screening with the fluorescent Hoechst 33256 stain [Chen, T.R., Exp. Cell Res., 104, 255-262 (1977)].

preparation of MSF

Confluent fetal and breast cancer patient skin fibroblasts cultured in 90 mm plastic dishes were washed 5 times with serum-free growth medium (SF-MEM) and then incubated with 5 ml SF-MEM for 72 hr at 37°C in a humidified incubator gassed with 5% CO_2

in air. The resultant conditioned medium (CM) was then collected and passed through a 0.22u Millipore filter to remove cellular debris and stored at -70°C until required.

We have previously demonstrated that all MSF activity in CM is adsorbed to a heparin affinity column and elutes at 0.3-0.6 M NaCl. Accordingly, 1 ml aliquots of CM were adsorbed to an immobilized heparin affinity column (4% beaded agarose with 0.4-0.5 mg/ml heparin; from Pierce Biochemicals, Chester, UK) previously equilibrated with 20 mM Tris-HCl/ 0.1 M NaCl (pH 7.0). The column was then washed with 5 ml of equilibrating buffer and MSF activity desorbed by stepwise elution with 0.3 and 0.6 M NaCl in 20 mM Tris-HCl (pH 7.0) until all protein was eluted, as estimated by A_{280} (ie. approximately 5 ml). Pooled 0.3 M NaCl column eluates were concentrated 10x by ultrafiltration using an Amicon filtration unit fitted with a YM10 Diaflo membrane (Amicon Ltd, Gloucestershire, UK). The resultant concentrated material was then fractionated by gel filtration chromatography using a Pharmacia fast protein liquid chromatography system (FPLC ^{tm}) as described in Example 1. This involved applying 200 ul aliquots to a Superose-12 column and running it at a flow rate of 0.3 ml/min in 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl. One ml fractions were collected and then dialyzed against SF-MEM for 48 hr. A peak of MSF activity eluted with a retention volume of 12-13 ml (V_o = 6.5 ml, V_t = 25 ml) in samples so processed from fetal and cancer patient skin fibroblast CM. This material (MSF) was freeze dried, stored at -25°C until required, and reconstituted in SF-MEM prior to use.

cell migration assay

Type I collagen was extracted from rat tail tendons in 3% acetic acid, dialyzed for two days against distilled water and used to make 2 ml collagen gels in 35 mm plastic tissue culture dishes (Schor and Court 1979). For our standard migration assay, collagen gels were overlaid with 1 ml of either SF-MEM (controls), neat CM or SF-MEM containing 40 ng/ml MSF. FSF37 fibroblasts were used as target cells in the assay since they do not produce MSF, but are responsive to it. Confluent stock cultures of FSF37 foreskin fibroblasts were trypsinized and resuspended in growth medium containing 20% serum. This cell suspension was then used to prepare a high and low density plating inoculum; the cell count in the high density inoculum was adjusted so that plating 1 ml gave 2.5×10^4 cells cm^{-2} on the collagen gel surface (ie. confluent density), whilst that in the low density inoculum was adjusted to give 10^3 cells cm^{-2} (ie. subconfluent density). Considering the 2 ml volume of the collagen gel, this procedure gives final concentrations of 5% serum in all cultures, and either 25% CM or 10 ng/ml MSF in the respective test cultures. Migration data are expressed as the percentage of fibroblasts found within the three-dimensional gel matrix after four days on incubation. These values were determined by counting the number of cells on the gel surface and within the collagen matrix in 10-15 randomly selected fields using a Leitz Labovert microscope (Schor 1980). A minimum of 500 cells were counted in low density cultures, whilst greater than 1000 cells were counted in high density cultures. Replicate gels were counted for each percentage point.

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CLAIMS

1. A migration stimulating factor.

2. Migration stimulating factor-1 which is a polypeptide capable of stimulating migration of normal adult fibroblasts which do not themselves produce the polypeptide and having an apparent molecular weight of 70kD by polyacrylamide gel electrophoresis, which is cationic at physiological pH, is precipitated from aqueous solution by ammonium sulphate at 10% saturation, which is stable in solution at pH 2 but not at pH 10, is denatured at 56°C and is susceptible to trypsin and alkylation/reduction and binds to heparin.

3. A polypeptide capable of stimulating migration of normal adult fibroblasts which do not themselves secrete a migration stimulating factor, having an apparent molecular weight of 50 to 70kD by gel filtration, which is stable in solution at pH2 but not at pH10, is denatured at 56°C and is suspectible to trypsin and alkylation/reduction.

4. A migration stimulating factor or polypeptide according to any one of claims 1 to 3 for use in a method for treatment of the human or animal body or in a method of diagnosis practised on the human or animal body.

5. A pharmaceutical composition comprising a migration stimulating factor or polypeptide according to any

one of claims 1 to 3 and a pharmaceutically acceptable carrier or diluent therefor.

6. A test or assay comprising contacting cells suspected to be susceptible to migration stimulating factors with a migration stimulating factor or polypeptide according to any one of claims 1 to 3.

7. A test or assay comprising contacting a sample suspected to contain a migration stimulating factors or polypeptide according to one of claims 1 to 3 with cells susceptibe to migration stimulating factors.

8. A test or assay according to claim 6 or claim 7 wherein the migratory behaviour of the cells is assessed by measuring the cell density migration index.

9. Antibodies against a migration stimulating factor or a polypeptide according to any one of claims 1 to 3.

10. A test or assay comprising contacting a sample suspected to contain a migration stimulating factor or a polypeptide according to any one of claims 1 to 3 with an antibody according to claim 9.

Fig. 1.

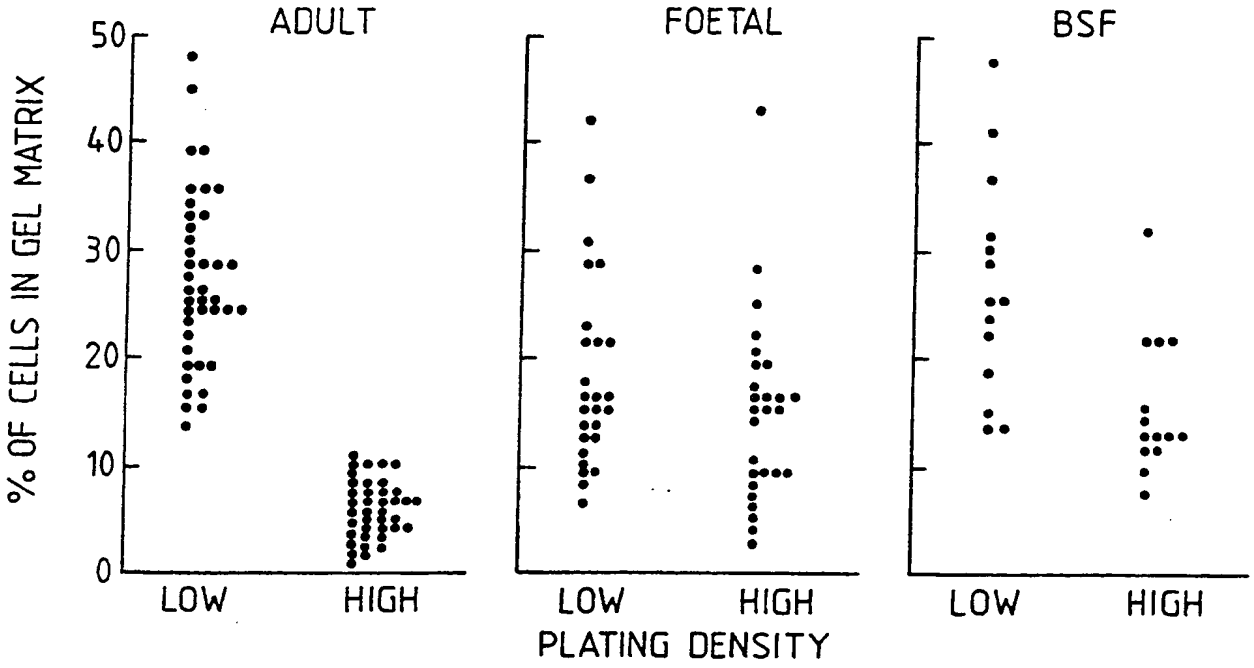
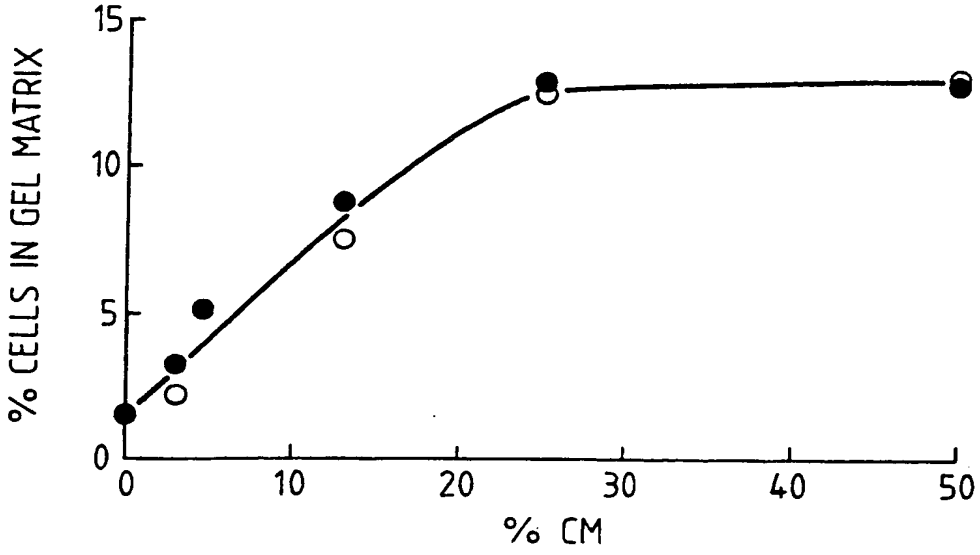


Fig. 2.



SUBSTITUTE SHEET

Fig.3.

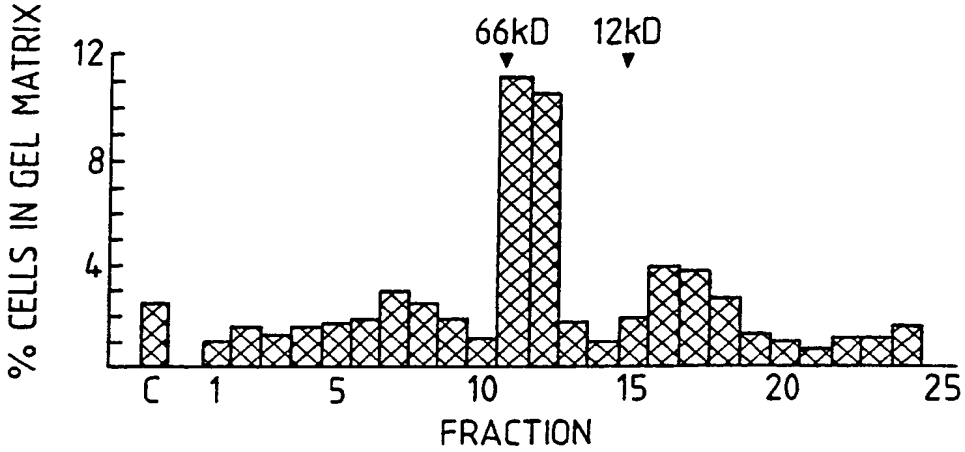
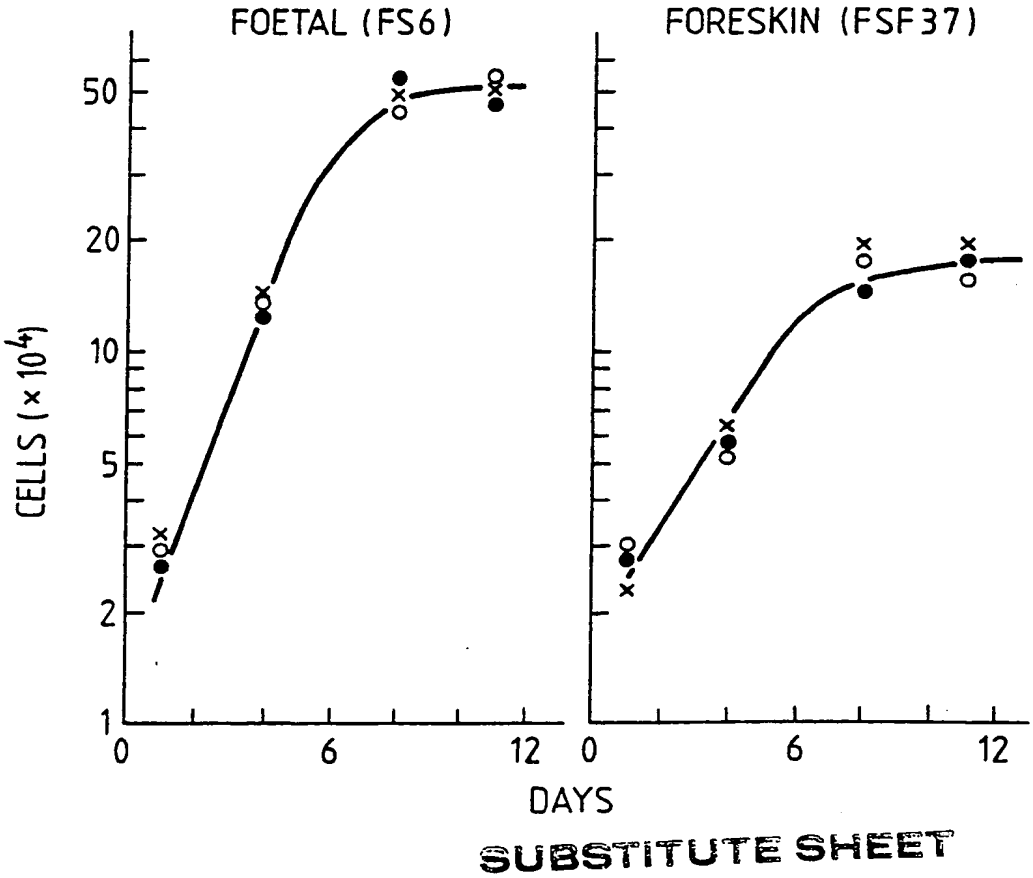


Fig.4.



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00768

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 15/06, A 61 K 37/02, C 12 Q 1/02, G 01 N 33/577 //
(A 61 K 37/02, 37:66)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC5	C 07 K; A 61 K; C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	Proc.Natl.Acad.Sci., Vol. 86, 1989 (USA) Anne-Marie Grey et al: "Purification of the migration stimulating factor produced byfetal and breast cancer patient fibroblasts ", see page 2438 - page 2442 --	1-10
X	Journal of Cell Science, Vol. 90, 1988 (Great Britain) Seth L. Schor et al: "Foetal and cancer patient fibroblasts produce an autocrine migrationstimulating factor not made by normal adult cells ", see page 391 - page 399 --	1-10
X	NATURE, Vol. 327, 1987 Michael Stoker et al: "Scatter factor is a fibroblast-derived modulator of epithelial cell mobility ", see page 239 - page 242 --	1

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29th September 1989	16 OCT 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Proc.Natl.Acad.Sci., Vol. 83, 1986 (USA) Lance A. Liotta et al: "Tumor cell autocrine motility factor ", see page 3302 - page 3306 --	1
X	Cancer Research, Vol. 45, 1985 Michael L. Basara et al: "Stimulation of Haptotaxis and Migration of Tumor Cells by Serum Spreading Factor ", see page 2487 - page 2494 --	1
X	J.Cell Sci., Vol. 77, 1985 (Great Britain) Michael Stoker and Marion Perryman: "An epithelial scatter factor released by embryo fibroblasts ", see page 209 - page 223 --	1
